CYCLOSPORINE A INHIBITS LYMPHOCYTE MIGRATION INTO OVINE PERIPHERAL NERVE ALLOGRAFTS

GREGORY M.T. HARE, M.D., Ph.D.,[1]
SUSAN E. MACKINNON, M.D.,[2]* RAJIV MIDHA, M.D., M.Sc.,[3]
PUI-YEUN WONG, Ph.D.,[4] BIHN AU, B.Sc.,[3]
CATHERINE MUNRO, M.Sc.,[3] WILLIAM ANDRADE, Ph.D.,[3]
DANIEL A. HUNTER, R.T.,[2] and JOHN B. HAY, Ph.D.[3]

Lymphocyte migration into nerve allografts was measured to estimate the cyclosporine A (CsA) dose required to suppress rejection. Twelve outbred sheep received daily subcutaneous CsA at 0, 5, 10, or 15 mg/kg/day for 2 weeks prior to implantation of multiple heterotopic subcutaneous nerve grafts. Lymphocyte migration was determined after 7 days by an intravenous pulse of autologous ^{111}indium-labeled lymphocytes and subsequent quantitation of gamma radioactivity in nerve tissue (CPM/g, mean ± SEM). Measurement by radioimmunoassay revealed a dose-dependent increase in blood cyclosporine levels. Lymphocyte migration into autografts (404 ± 44) was significantly less than migration into allografts (16,554 ± 2,049), in control animals ($P < 0.01$). A dose-dependent inhibition of lymphocyte migration into nerve allografts was observed with counts of 7,662 ± 1,692, 4,083 ± 1,112, and 1,561 ± 232 in sheep receiving 5, 10, or 15 mg/kg/day of CsA, respectively. Daily CsA administration produced effective blood levels and immunosuppression sufficient to inhibit lymphocyte migration into nerve allografts.

MICROSURGERY 17:697–705 1996

Nerve allografts have been utilized clinically to graft otherwise irreparable cases of peripheral nerve injuries.[1,2] Systemic immunosuppression has been utilized to prevent rejection and to allow optimal axonal regeneration across nerve allografts experimentally[3–6] and clinically.[1,2] However, potential toxicity associated with long-term cyclosporine A (CsA) treatment must be balanced with the potential for axonal regeneration across nerve allografts and neurological recovery. Attempts to reduce nerve allograft antigenicity by various pretreatment methods, including irradiation and lypophilization, have produced only limited success.[7–9] Cold preservation decreased antigenicity of nerve allografts in sheep suppressing early immunological events[10–12]; however, axonal regeneration across long nerve allografts was not supported in the absence of systemic immunosuppression.[13]

The optimal requirements for systemic immunosuppression of peripheral nerve allografts have not been clearly evaluated in a large animal model. By contrast, numerous studies have evaluated recovery over short nerve gaps.[3–6] In rats, temporary CsA immunosuppression has been shown to be as effective as continuous CsA treatment in supporting regeneration across short tibial nerve allografts.[14] It is unknown if this is the case for reconstruction of larger nerve allografts. In primates, axonal regeneration across a 3 cm ulnar nerve allograft occurred equally in the presence and absence of CsA immunosuppression, suggesting that such a nerve gap was of insufficient length to challenge axonal regeneration.[15]

Sheep have a well-characterized immune system[16,17] and tolerate long nerve gaps with minimal morbidity.[11,13] CsA administered via gastric fistula or intravenous route has been previously studied in sheep.[18–20] The purpose of this study was to develop a model of subcutaneous CsA administration in sheep. Pharmacological drug measurements will ensure adequate drug levels in the blood. Inhibition of lymphocyte migration into nerve allograft segments and delayed-type hypersensitivity (DTH) skin lesions will serve as a biological marker of systemic immunosuppression. These data will be used to determine the minimal systemic CsA dose that may support axonal regeneration across long peripheral nerve allografts in future studies.

MATERIALS AND METHODS

Animal Protocol

Twelve outbred sheep were primed intradermally with bacillus Calmette-Guérin (BCG) vaccine (2.5 doses/animal;

[1]Department of Anaesthesia, Dalhousie University, Halifax, Nova Scotia, Canada

[2]Division of Plastic Surgery, Barnes Hospital, St. Louis, MO

[3]Departments of Surgery and Immunology, University of Toronto, Trauma Research Program, Sunnybrook Health Sciences Centre, North York, Ontario M4N 3M5, Canada

Grant sponsor: NIH; Grant number: #5ROI NS33406-02; Grant sponsor: MRC Canada Support.

*Correspondence to: Susan E. Mackinnon, Division of Plastic Surgery, Suite 17424, One Barnes Hospital Plaza, St. Louis, MO 63110.

Connaught Laboratories, Willowdale, Ontario, Canada) 2 weeks prior to initiating experiments. Animals were acclimatized to the surroundings and baseline weights, and renal and hepatic function was established prior to initiating daily CsA administration. Liquid CsA (100 mg/ml in olive oil vehicle; Sandimmune, Novartis Pharma Inc., Canada; Dorval, Quebec, Canada) was administered daily in rotating subcutaneous sites along the back of the animal in doses of 0, 5, 10, and 15 mg/kg/day (n = 4, 2, 4, and 2 animals/group, repectively).[21,22] Control animals received an equivalent volume of drug vehicle alone. Daily blood samples were drawn for cyclosporine level determination, and weekly blood samples were drawn to assess renal and hepatic function. Drug doses were adjusted according to weekly weights. Animals were kept in pens with access to food and water ad libitum and cared for in accordance with the guidelines of the University of Toronto Animal Care Committee.

After 2 weeks of CsA administration, animals were fasted overnight prior to general anesthesia. After Pentothal induction (1.25%, 10 mg/kg IV; Abbott, Montreal, Quebec, Canada) and endotracheal intubation, general anesthesia was maintained by spontaneous ventilation with halothane (Halocarbon Laboratories, North Augusta, SC) and oxygen. Bilateral median nerves were sequentially exposed via a medial forearm incision, and 10 cm of a predominantly sensory branch of the median nerve was excised. Peroneal nerves were harvested by a lateral incision as described previously.[10] Peroneal nerve allograft donors were euthanized following nerve harvest (Euthanol, 1 ml/kg IV; MTC Pharmaceuticals, Cambridge, Ontario, Canada). The nerves were then subdivided into 1 cm segments and labeled with 3.0 nylon sutures (American Cyanamid, Pearle River, NY). Multiple (n = 5 or greater) median autografts, median allografts (donor #1), and peroneal allografts (donor #2) were implanted in separately labeled subcutaneous pockets on a single flank of each animal. All wounds were closed with subcuticular 3.0 absorbable suture. Animals were allowed to recover and received postoperative analgesia (Temgesic, Buprenorphine (Beckitt and Coleman Products, Hull, England) 0.005 mg/kg IM q6h). All animals were walking freely in pens 4 hours postoperatively. Nerve graft segments were left in situ for 7 days prior to lymphocyte migration studies. Daily CsA administration was continued during this time.

On postoperative day 5, animals were again anesthetized for DTH skin lesions and efferent prescapular lymphatic cannulation. Subscapular efferent lymphatics were exposed by a lateral neck incision and cannulated with flexible polyethylene tubing as previously described.[17] Cannulae were then fixed in place with silk ligatures and lymph collection initiated. On postoperative day 7, 5×10^8 autologous efferent lymphocytes were collected and labeled with ^{111}indium oxine prior to intravenous injection. After 3 hours of recirculation the animals were euthanized and nerve tissue and skin sites harvested.

Nerve grafts were placed in preweighed glass test tubes and the wet weight obtained before counting in a gamma counter and expressed as CPM/g tissue. Animal skins were frozen (−70°C) and a 1 cm punch was used to harvest skin sites. Individual skin sites were placed in test tubes and gamma counts expressed as CPM/skin site.

Delayed-Type Hypersensitivity Skin Lesions

All intradermal injections were performed with animals anesthetized prior to cannulation of an efferent prescapular lymphatic for lymphocyte harvesting. DTH skin lesions were initiated 5 days after nerve implantation on the contralateral flank. Purified protein derivative (PPD) stock solution (Connaught Laboratories, Willowdale, Ontario, Canada) was diluted with 2% Evans blue saline solution to final concentrations of 20 or 60 μg/0.2 ml and injected intradermally (n = 6 sites/dose). Control injection sites contained Evans blue saline and/or CsA vehicle. The effect of local CsA (1.0 mg/site) was determined by concomitant injection with 20 μg of PPD/site (n = 6 sites). All DTH lesions were allowed to develop for 48 hours, at which time the maximum response was noted.[17]

Lymphocyte Preparation

Two days prior to assessment of lymphocyte migration, each animal underwent a second general anesthetic, as outlined above, and a single efferent prescapular lymphatic was cannulated as described previously.[17] The cannulae were maintained for 2 days prior to lymphocyte migration studies, which were performed 7 days after nerve graft implantation and 48 hours after DTH lesion injections. These times correspond to the maximum effect on lymphocyte migration.[10,17] Autologous lymphocytes were collected into a penicillin/heparin solution over a 6 hour period prior to labeling. Using a Coulter particle counter (model 901, Coulter Electronics, Hialeah, FL) 5×10^8 cells were aliquoted for labeling. Cells were washed 3 times in Hanks' balanced salt solution (HBSS) and suspended to a concentration of 10^8 cells/ml. To this suspension, ^{111}indium oxine (Amersham, Oakville, ONT, Canada) was added at a concentration of 10 μCi/10^8 cells. After incubation at room temperature for 10 minutes, 10 ml of lymph plasma was added to bind any free ^{111}indium. The cells were then washed in HBSS and resuspended in fresh lymph plasma. The radiolabeled cells were then injected intravenously and allowed to recirculate for 3 hours.

Histology

At the time of tissue harvest, segments of median nerve in situ and median autograft and allograft segments were fixed by immersion in 3% gluteraldehyde in 0.2 M phosphate buffer (pH 7.4) to which H_2O_2 was added (5 μl/10 ml

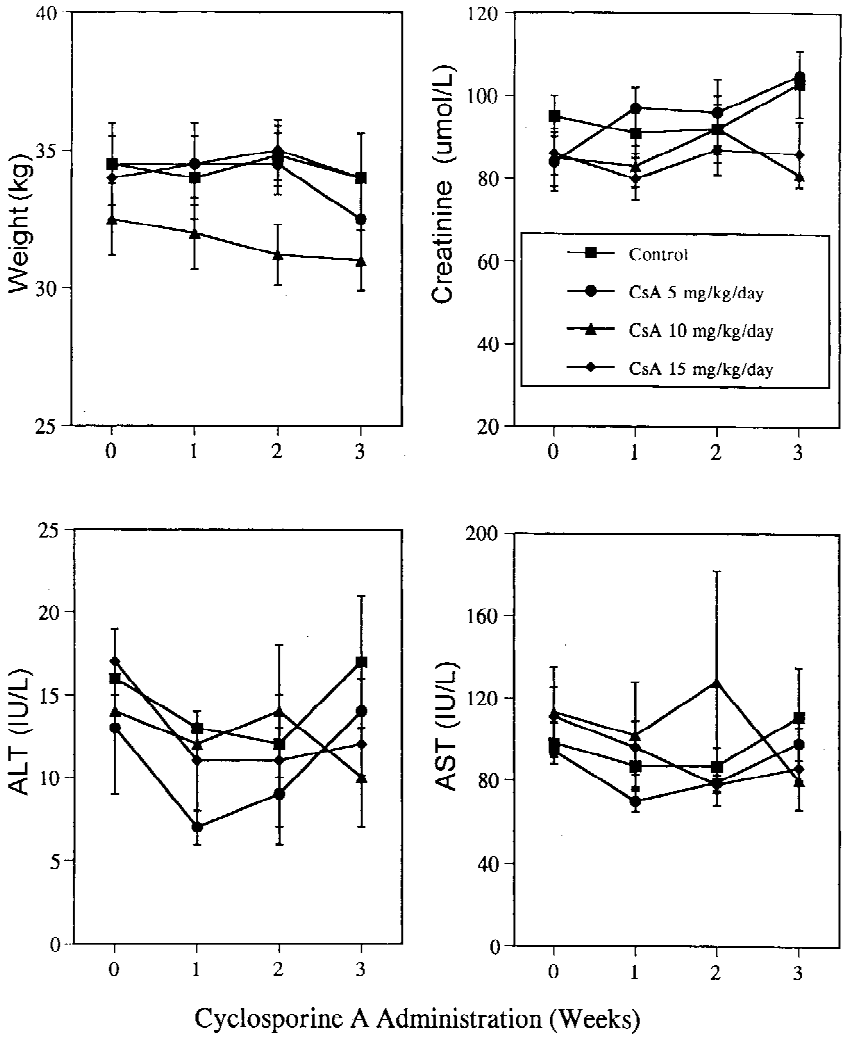

Figure 1. Weekly body weights, creatinine, alanine transferase (ALT), and aspartate transferase (AST) remained stable during subcutaneous CsA administration without any indication of systemic toxicity.

analytic grade, BDH, Toronto, Ontario, Canada). After 1 hour, the nerve segments were transferred to fresh gluteraldehyde.[23] Postfixation was performed with osmium tetroxide, and the the nerves were imbedded in Araldite 502 (Polysciences, Warrington, PA). Toluidine blue was used to stain 1 μm sections of nerve tissue cut with an LKBIII ultramicrotome (LKB-Produkter, Bromma, Sweden) for light microscopy.

Histomorphometric Analysis

Quantitative lymphocyte counts were performed on median nerve allograft segments in sheep receiving 0, 5, 10, and 15 mg/kg/day CsA using light microscopy. At 1,000× magnification, seven representative fields were evaluated using a digital image analysis system linked to morphometry software (Leco Instruments, St. Joseph, MI). For each nerve segment, extraneural lymphocyte nuclei were counted. For each nerve segment the total surface area measured was constant. Results are reported as total lymphocytes counted for a constant total surface area.

Biochemical Measurements

Serum aspartate transferase (AST), alanine transferase (ALT), and creatinine were measured prior to initiating CsA and then weekly. Whole blood trough cyclosporine levels were measured dialy using a specific monoclonal radioimmunoassay (Cyclotrac, INCSTAR, Stillwater, MN), calibrated with CsA whole blood standards.[24]

Statistial Analysis

A power analysis was performed with results suggesting that six animals/drug dose group would be required to obtain statistically significant differences in lymphocyte migration. Due to overall cost restrictions, n = 4, 2, 4, and 2 animals were used for CsA doses of 0, 5, 10, and 15 mg/kg/day, respectively, acknowledging the potential for compromised power of statistical analysis.

All results are reported as mean ± SEM. An analysis of variance model (ANOVA) was applied to account for the variable sample size. A Dunnett's test was used for multiple comparisons to the control group.[25,26] Comparison of two

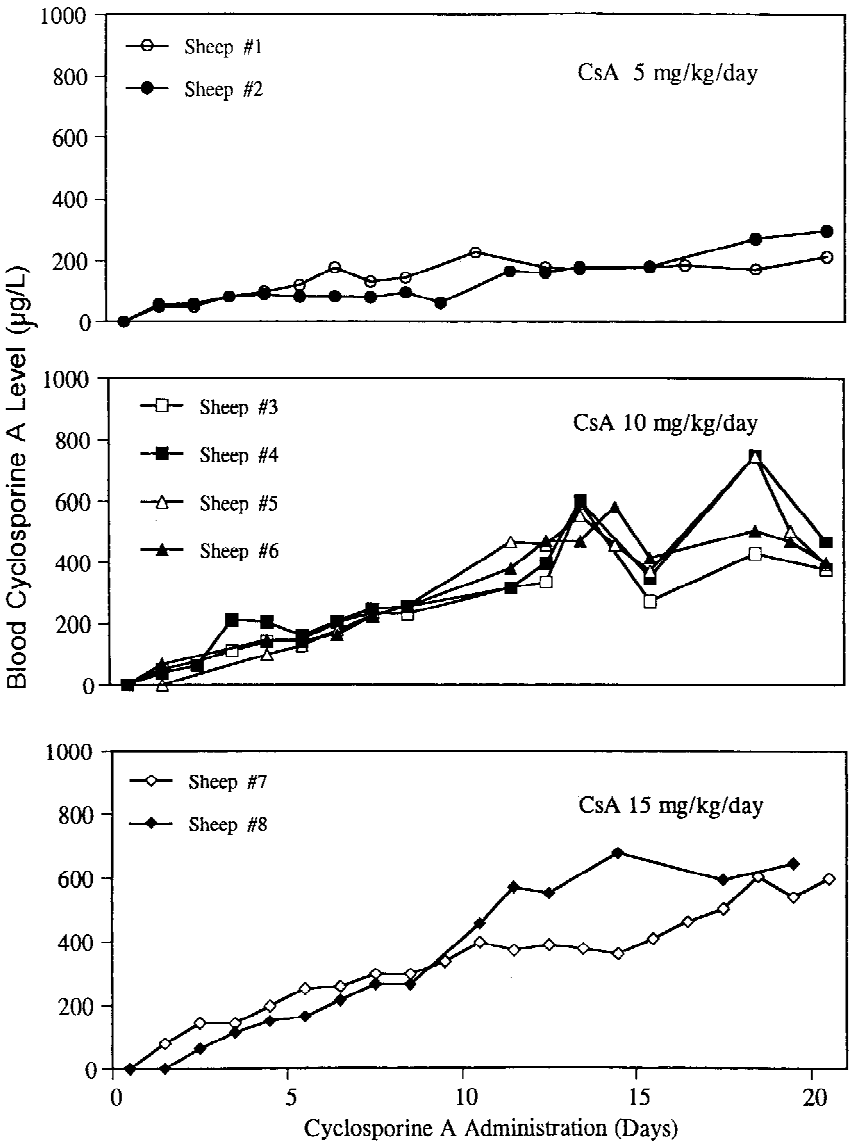

Figure 2. Blood cyclosporine levels approached plateau values near 250, 400, and 600 µg/L after 2 weeks of daily CsA administration at 5, 10, or 15 mg/kg/day, respectively.

means was performed using Student's t-test. A *P* value (two-tailed) of <0.05 was taken as significant.

RESULTS

Weight and Renal and Hepatic Function

All animals tolerated the protocol without evidence of systemic CsA toxicity as determined by animal weights and renal and hepatic function (Fig. 1).

Blood Cyclosporine Levels

Blood cyclosporine was not detected in any of the control animals over the duration of the experiment. In all experimental groups, cyclosporine levels increased steadily over the first 2 weeks. During the third week, cyclosporine

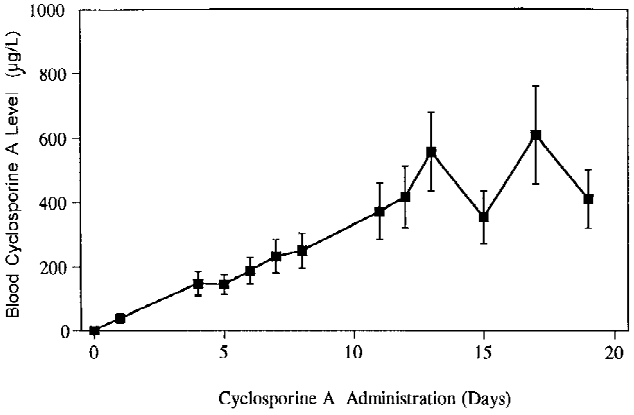

Figure 3. Mean blood cyclosporine levels plateau after 2 weeks of CsA administration at 10 mg/kg/day (mean ± SEM; n = 4 sheep).

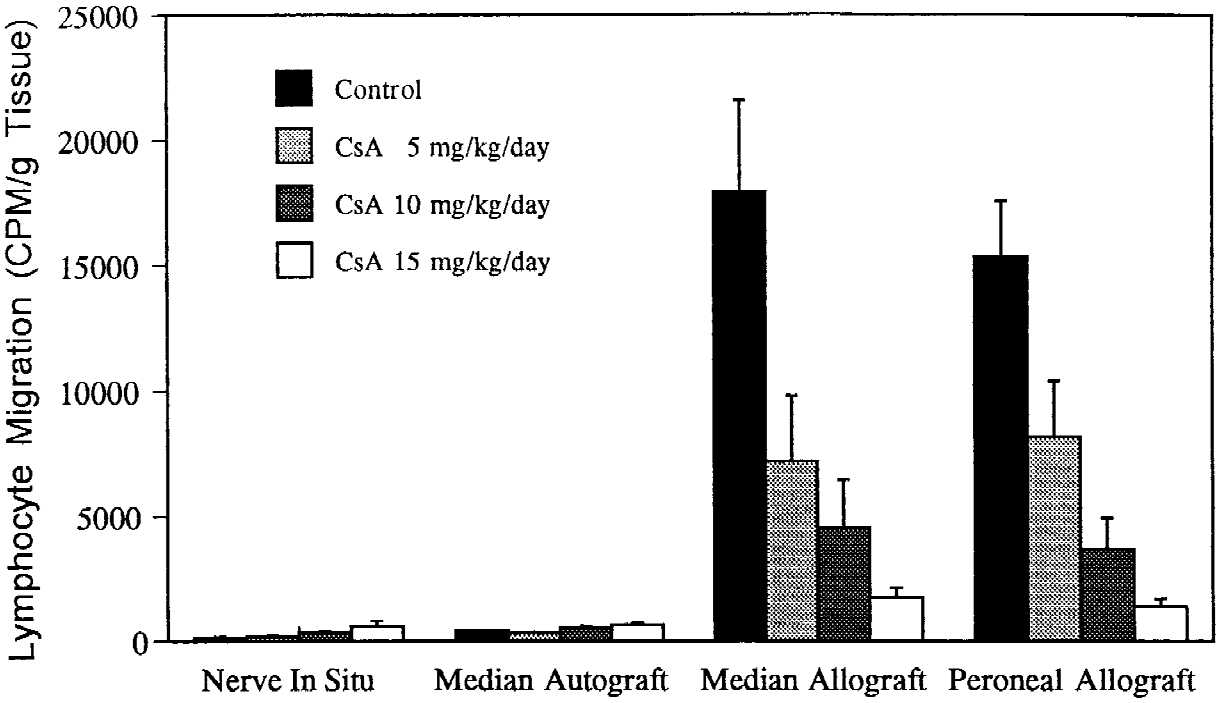

Figure 4. Radiolabeled lymphocyte migration into nerve in situ and median autograft remain consistently low in control and CsA groups. Lymphocyte migration into median and peroneal nerve allografts is substantially increased in control animals, relative to autograft values (black bars). Systemic CsA inhibits lymphocyte migration into both allografts in a dose-dependent manner, with the most profound effect at 15 mg/kg/day (open bars).

levels approached plateaus of approximately 250, 400, and 600/L in the 5, 10, and 15 mg/kg/day groups, respectively (Figs. 2, 3).

Lymphocyte Migration Into Nerve Allografts

Lymphocyte migration into unmanipulated nerves in situ was negligible with values of 145 ± 48, 188 ± 61, 341 ± 45, and 601 ± 210 CPM/g tissue after 0, 5, 10, and 15 mg/kg/day of CsA, respectively (n = 17, 8, 16, and 9 nerve segments/group, respectively) (Fig. 4). Similarly, median nerve autografts had low lymphocyte migration values of 404 ± 44, 312 ± 39, 507 ± 95, and 638 ± 103 CPM/g tissue after 0, 5, 10, and 15 mg/kg/day of CsA, respectively (n = 19, 9, 17, and 8 grafts/group, respectively) (Fig. 4).

Lymphocyte migration into peroneal and median nerve allografts from two different donors was dramatically increased above controls (Fig. 4). Pooling median and peroneal nerve allograft samples in the control group (CsA 0 mg/kg/day) yielded a lymphocyte migration value of 16,554 ± 2049, which was significantly greater than the comparable autograft value of 404 ± 44 ($P < 0.01$). Administration of CsA resulted in a dose-dependent reduction in lymphocyte migration into nerve allografts, with the most predominant reduction after 15 mg/kg/day (Fig. 4). Pooled median and peroneal nerve allograft results for each group yielded lymphocyte migration values of 7,662 ± 1,692, 4,083 ± 1,112, and 1,561 ± 232 CPM/g tissue after 5, 10, and 15 mg/kg/day of CsA, respectively (n = 45, 21, 36, and 24 grafts/group). Despite a very large effect (f = 0.955), the differences between groups as assessed by ANOVA were not statistically significant.

Histologic assessment of lymphocyte counts supports the data obtained using radiolabeled cells. Few lymphocytes were observed in light micrographs of pristine nerve in situ or nerve autografts (Fig. 5A,B). There was a predominant lymphocytic infiltrate in the control allografts (Fig. 5C). This decreased in a dose-related fashion with 5, 10, and 15 mg/kg/day CsA immunoosuppression (Fig. 5D–F). Again, the reduction in lymphocyte count with different doses of CsA did not reach statistical significance when assessed by ANOVA. Comparison of radioactive lymphocyte counts with quantitative histomorphometric lymphocyte counts approached a linear relationship (Fig. 6).

Lymphocyte Migration Into Delayed-Type Hypersensitivity Skin Lesions

Lymphocyte migration into DTH skin lesions showed a dose-related increase with 20 or 60 μg of PPD in control animals (Fig. 7, black bars). Systemic CsA did not affect this relationship nor did it reduce lymphocyte migration into DTH lesions at doses of 5 and 10 mg/kg/day (Fig. 7, light and dark gray bars). At 15 mg/kg/day, CsA inhibited lymphocyte migration into DTH lesions initiated with 20 or 60 μg PPD/lesion (Fig. 7, white bars), although this did not reach statistical significance when assessed by ANOVA. In the absence of systemic CsA, local CsA (1 mg/site) partially

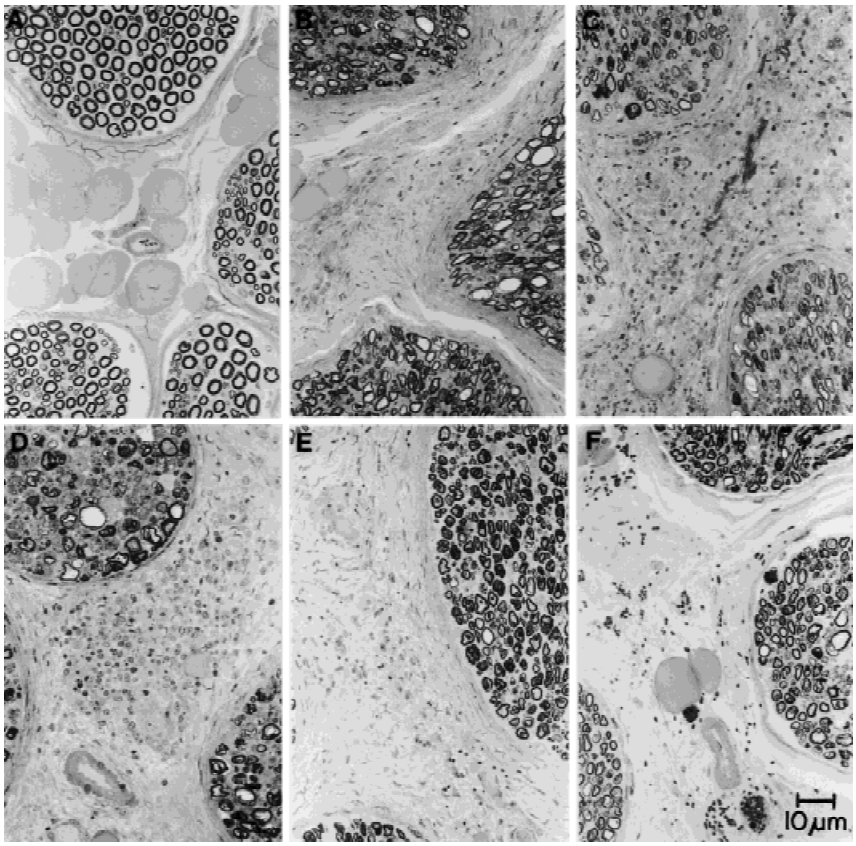

Figure 5. ***A:*** *Median nerve in situ with intact perineurium and few migrant cells.* ***B:*** *Median nerve autograft demonstrating intact fascicular structure and few infiltrating large mononuclear cells.* ***C:*** *Median nerve allograft in control animals demonstrating extensive infiltration of predominantly small mononuclear lymphocytes and destruction of fascicular architecture.* ***D:*** *Systemic CsA at 5 mg/kg/day reduces the small mononuclear cell infitrate into the median nerve allograft.* ***E:*** *Median nerve allograft treated with CsA 10 mg/kg/day shows a more profound effect on lymphocyte migration.* ***F:*** *Median nerve allograft tested with CsA 15 mg/kg/day shows very little mononuclear cell infiltrate.*

inhibited lymphocyte migration into DTH lesion initiated with 20 μg PPD/site (Fig. 8) ($P < 0.01$).

DISCUSSION

Systemic CsA has supported life-sustaining organ transplantation.[27–30] This therapy has also been utilized in the treatment of a number of immune-mediated diseases including diabetes mellitus,[31] psoriasis,[32] rheumatoid arthritis,[33] and inflammatory bowel disease.[34] Administration of CsA to nerve transplant recipients in cases of severe peripheral nerve injuries has prolonged nerve allograft survival and significantly reduced lifelong morbidity in trauma patients.[1,2] Experimental evidence suggests that temporary CsA therapy is as effective as continuous therapy, in supporting axonal regeneration across short nerve allografts.[14] Furthermore, discontinuation of systemic immunosuppression several years after clinical nerve allograft implantation has not been associated with detrimental effects on neuro-

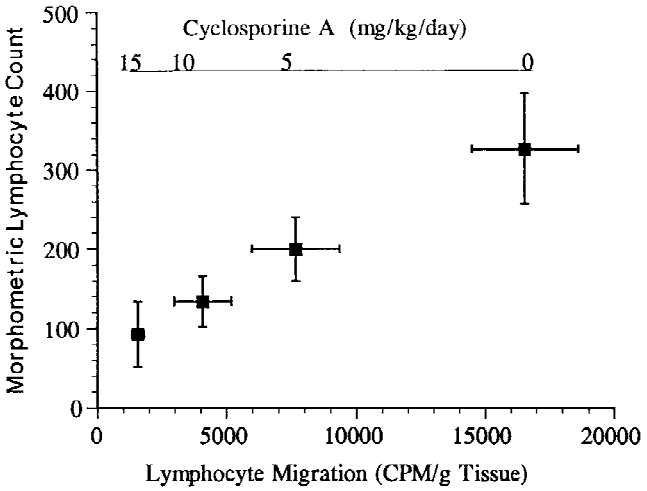

Figure 6. The linear relationship between quantitative assessment of lymphocyte migration by ^{111}indium labeling and histomorphometric cell counts show good correlation of these two methods ($r = 0.9285$).

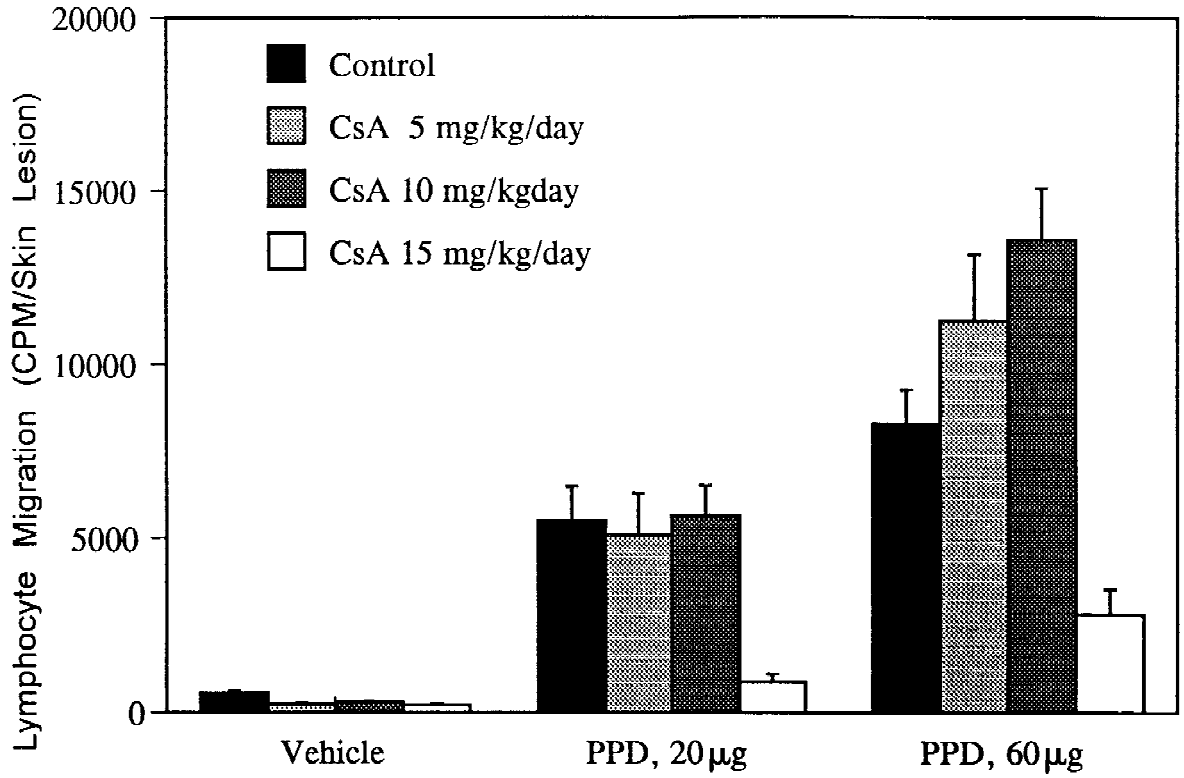

Figure 7. Lymphocyte migration into DTH skin lesions induced by 20 and 60 μg of PPD is inhibited by the highest dose of CsA (15 mg/kg/day).

logical improvements.[1,2] However, the optimal duration and dose of CsA immunosuppression for peripheral nerve transplantation have not been clearly defined in a large animal model or clinically. As a first step toward this goal, an ovine model was developed to evaluate the optimal dose of CsA required to initiate systemic immunosuppression for nerve transplantation. Lymphocyte migration into heterotopic subcutaneous nerve allografts and DTH skin lesions were used as biological markers of systemic immunosuppression and correlated with blood cyclosporine levels.

Although subcutaneous administration of CsA has been utilized in rat sciatic nerve transplantation,[21,22] this method of CsA administration has not been documented in sheep. Published studies in sheep have utilized intravenous or direct injection into the glandular stomach via gastric fistula to avoid the variable absorption associated with the ovine ruminant stomach.[18–20] Intravenous CsA injection has also been associated with variable daily drug levels.[35] For long-term studies, subcutaneous CsA administration would require less intravention and hence be more practical and advantageous.

Daily subcutaneous administration of CsA of 5, 10, or 15 mg/kg/day established blood levels comparable to those used in clinical studies for renal, liver, and heart transplants.[28–30] Drug levels reached a plateau during the third week of treatment. Regular monitoring of cyclosporine levels in sheep, similar to that done in clinical practice,[27] would be required in long-term studies to limit dose requirements and reduce the risk of systemic toxicity.

As previously reported,[10] radiolabeled lymphocyte migration into control peripheral nerve allografts receiving no CsA was, on average, 40-fold greater than autograft values. With CsA administration, there was a dose-dependent decrease in lymphocyte migration into nerve allografts, with maximum inhibition using 15 mg/kg/day of CsA. At lower doses (5 and 10 mg/kg/day), persistent lymphocyte migration occurred into nerve allographs despite reasonable CsA blood levels. This may reflect the finding that, at clinically relevant blood levels, systemic CsA only partially inhibits the cellular mechanisms involved in immunosuppression, such as calcineurin phosphate activity and subsequent activation of cytokine transcription.[36]

By comparison, lymphocyte migration into DTH skin lesions was not inhibited until the maximal dose of 15 mg/kg/day of CsA was utilized, perhaps because animals were initially sensitized with BCG prior to initiating immunosuppression. Furthermore, CsA has been paradoxically shown to enhance the DTH reaction in mice,[37] possibly explaining why a higher dose of CsA was required to inhibit lymphocyte migration in DTH lesions in sheep. Pronounced inhi-

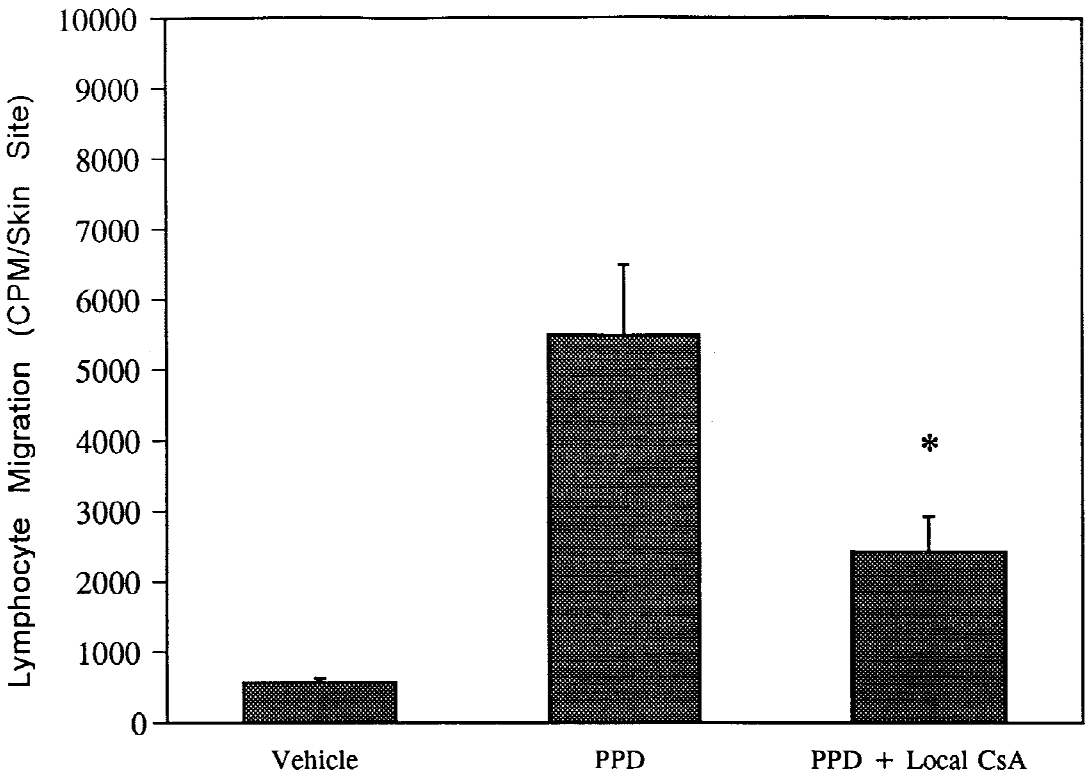

Figure 8. Local CsA (1 mg/skin site) inhibits lymphocyte migration into DTH skin lesions (PPD 20 µg/skin site) in control animals with no systemic CsA ($P < 0.01$).

bition of lymphocyte migration into allografts and DTH lesions by 15 mg/kg/day of CsA suggests that this may be a good starting dose to allow axonal regeneration across nerve allografts.

Histological assessment of nerve allograft segments confirm the quantitative lymphocyte migration data. Progressively fewer invading lymphocytes are observed with increasing doses of CsA. Analysis of the number of lymphocytes per microscopic field correlated well with the values obtained using ^{111}indium-labeled lymphocytes. Additionally, local CsA significantly reduced local lymphocyte migration into DTH skin lesions. Although local administration of CsA was not applied to nerve allografts, it may support development of local CsA treatment as a means of limiting CsA toxicity.

Systemic immunosuppression using subcutanoues CsA in sheep provides a model for studying axonal regeneration across long nerve allografts. Utilization of median nerve grafts in these animals provides an internally controlled model for studying axonal regeneration across nerve allografts. The contralateral nerve autograft would provide the gold-standard comparison. This model would also permit determination of the optimal dose of CsA required for systemic immunosuppression. The median nerve grafting technique is well tolerated with minimal morbidity and provides the potential for long (10 cm) nerve engraftment. Future studies will utilize these results to determine the conditions of optimal axonal regeneration across long nerve grafts in sheep.

ACKNOWLEDGMENTS

We thank Novartis Pharma Canada Inc. for their generous supply of cyclosporine A, Dr. J.P. Szalai and Mr. Marco Katic for their expert statistical advice, and Mel Walker and Diana Armstrong for their technical assistance.

REFERENCES

1. Mackinnon SE, Hudson AR: Clinical application of peripheral nerve transplantation. *Plast Reconstr Surg* 90:695–699, 1992.
2. Mackinnon SE: Nerve allotransplantation following severe tibial nerve injury. *J Neurosurg* 84:671–676, 1996.
3. Bain JR, Mackinnon SE, Hudson AR, Falk RE, Falk JA, Hunter DA: The peripheral nerve allograft: An assessment of regeneration across nerve allografts in rats immunosuppressed with cyclosporin A. *Plast Reconstr Surg* 82:1052–1064, 1988.
4. Yu LT, England J, Sumner A, Larossa D, Hickey WF: Electrophysiological evaluation of peripheral nerve regeneration through allografts immunosuppressed with cyclosporin. *J Reconstr Microsurg* 6:317–323, 1990.
5. Gulati AK: Evaluation of acellular and cellular nerve grafts in repair of rat peripheral nerve. *J Neurosurg* 68:117–123, 1988.

6. Zalewski AA, Gulati AK: Survival of nerve allografts in sensitized rats treated with cyclosporin A. *J Neurosurg* 60:828–834, 1984.
7. Ide C, Osawa T, Tohyama K: Nerve regeneration through allogenic nerve grafts, with special reference to the role of the Schwann cell basal lamina. *Prog Neurobiol* 34:1–38, 1990.
8. Mackinnon SE, Hudson AR, Falk RE, Kline D, Hunter D: Peripheral nerve allograft: An immunological assessment of pretreatment methods. *Neurosurgery* 14:167–171, 1984.
9. Zalewski AA, Gulati AK: Evaluation of histocompatibility as a factor in the repair of nerve with a frozen nerve allograft. *J Neurosurg* 56: 550–554, 1982.
10. Hare GMT, Evans PJ, Mackinnon SE, Nakao Y, Midha R, Wade JA, Hunter DA, Hay JB: Effect of cold preservation on lymphocyte migration into peripheral nerve allografts in sheep. *Transplantation* 56: 154–162, 1993.
11. Hare GMT, Evans PJ, Mackinnon SE, Wade JA, Young AJ, Hay JB: Phenotypic analysis of migrant, efferent lymphocytes after implantation of cold preserved, peripheral nerve allografts. *J Neuroimmunol* 56:9–16, 1995.
12. Strasberg SR, Mackinnon SE, Hare GMT, Narini PP, Hertl MC, Hay JB: Reduction in peripheral nerve allograft antigenicity with warm and cold temperature preservation. *Plast Reconstr Surg* 97:152–160, 1996.
13. Strasberg SR, Mackinnon SE, Genden EM, Bain JR, Purcell CM, Hunter DA, Hay JB: Long-segment nerve allograft regeneration in the sheep model: Experimental study and review of the literature. *J Reconstr Microsurg* 12:529–537, 1996.
14. Midha R, Mackinnon SE, Evans PJ, Best TJ, Hare GMT, Hunter DA, Falk-Wade JA: Comparison of regeneration across nerve allografts with temporary or continuous cyclosporin A immunosuppression. *J Neurosurg* 78:90–100, 1993.
15. Bain JR, Mackinnon SE, Hudson AR, Wade JA, Evans PJ, Mikino AP, Hunter DA: The peripheral nerve allograft in the primate immunosuppressed with cyclosporin A: I. Histologic and electrophysiologic assessment. *Plast Reconstr Surg* 90:1036–1046, 1992.
16. Mackay CR: Summary of workshop findings for leukocyte antigens of sheep. *Vet Immunol Immunopathol* 27:28–32, 1991.
17. Borgs P, Hay JB: A quantitative lymphocyte localization assay. *J Leukocyte Biol* 39:333–342, 1986.
18. Charles BG, Filippich LJ, Pass MA: Pharmacokinetics and absolute bioavailability of cyclosporin following intravenous and abomasal administration to sheep. *J Pharm Pharmacol* 45:821–824, 1993.
19. Treshman JA, Whitworth JA, Scoggins BA, Bennett WM: The failure of nisodipine to prevent the hypertensive response to cyclosporin A infusion in sheep. *J Hypertens* 8:1007–1013, 1990.
20. Tresham JA, Whitworth JA, Scoggins BA, Bennett WM. Cyclosporine-induced hypertension in sheep. The role of thromboxanes. *Transplantation* 49:144–148, 1990.
21. Midha R, Mackinnon SE, Wade JA, Evans PJ, Best TJ, Wong PY: Chronic cyclosporine A therapy in rats. *Microsurgery* 13:273–276, 1992.
22. Midha R, Mackinnon SE, Evans PJ, Best TJ, Wong PY: Subcutaneous injection of oral cyclosporin A solution. *Microsurgery* 13:92–94, 1992.
23. Peracchia C, Mittler BS: Fixation by means of gluteraldehyde-hydrogen peroxide reaction products. *J Cell Biol* 53:234–238, 1972.
24. Wong PY, Ma J: Specific and nonspecific monoclonal ^{125}I-Incstar assays. *Transplant Proc* 2:1166–1170, 1990.
25. Dunnett CW: A multiple comparison procedure for comparing several treatments with a control. *J Am Stat Assoc* 50:1096–1103, 1955.
26. Winer BJ: *Statistical Principles in Experimental Design* (2nd ed). London, McGraw-Hill, 1971.
27. Kahan BD: Cyclosporine. *N Engl J Med* 321:1725–1738, 1989.
28. The Canadian Multicentre Transplant Study Group: A randomized clinical trial of cyclosporine in cadaveric renal transplantation: Analysis at three years. *N Engl J Med* 314:1219–1225, 1986.
29. Oyer PE, Stinson EB, Jamieson SW, Hunt SA, Perlroth M, Billingham M, Shumway NE: Cyclosporine in cardiac transplantation: A 2 1/2 year follow-up. *Transplant Proc* 15:2546–2552, 1983.
30. Starzl TE, Klintmalm GBG, Porter KA, Iwatsuki S, Schroter GPJ: Liver transplantation with use of cyclosporin A and prednisone. *N Engl J Med* 305:266–269, 1981.
31. Feutren G, Papoz L, Assan R, Vialettes B, Karstenv G, Vexiau P, Du-Rostu H, Rodier M, Sirmai J, Lallamand A, Back JF: Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset: Results of a multicentre double-blind trial. *Lancet* 2:119–124, 1986.
32. Zachariae H, Steen-Olsen T: Efficacy of cyclosporin A (CyA) in psoriasis: An overview of dose/response, indications, contraindications and side-effects. *Clin Nephrol* 43:154–158, 1995.
33. Flipo RM, Reigneau O: The benefit/risk ratio of low dose cyclosporin A in rheumatoid arthritis. *J Rheumatol* 23:404–405, 1996.
34. Korelitz BI: Immunosuppressive therapy of inflamatory bowel disease: A historical perspective. *Gastroenterologist* 3:141–152, 1995.
35. Wassef R, Cohen Z, Langer B: Pharmacokinetic profiles of cyclosporine in rats. Influence of route of administration and dosage. *Transplantation* 40:489–493, 1985.
36. Batiuk TD, Urmson J, Vincent D, Yatscoff RW, Halloran PF: Quantitating immunosuppression. Estimating the 50% inhibitory concentration for *in vivo* cyclosporine in mice. *Transplantation* 61:1618–1624, 1996.
37. Webster LM, Thomson AW: Cyclosporine A prevents suppression of delayed-type hypersensitivity in mice immunized with high-dose sheep erythrocytes. *Immunology* 60:409–415, 1987.